

Distinct Roles of the N-Terminal and C-Terminal Precursor Domains in the Biogenesis of the *Bordetella pertussis* Filamentous Hemagglutinin

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The 220-kDa *Bordetella pertussis* filamentous hemagglutinin (FHA) is the major exported protein found in culture supernatants. The structural gene of FHA has a coding potential for a 367-kDa protein, and the mature form constitutes the N-terminal 60% of the 367-kDa precursor. The C-terminal domain of the precursor was found to be important for the high-level secretion of full-length FHA but not of truncated analogs (80 kDa or less). The secretion of full-length and truncated FHA polypeptides requires the presence of the approximately 100-amino-acid N-terminal domain and the outer membrane protein FhaC, homologous to the N-terminal domains of the *Serratia marcescens* and *Proteus mirabilis* hemolysins and their accessory proteins, respectively. By analogy to these hemolysins, it is likely that the N-terminal domain of the FHA precursor interacts, directly or indirectly, with the accessory protein during FHA biogenesis. However, immunogenicity and antigenicity studies suggest that the N-terminal domain of FHA is masked by its C-terminal domain and therefore should not be available for its interactions with FhaC. These observations suggest a model in which the C-terminal domain of the FHA precursor may play a role as an intramolecular chaperone to prevent premature folding of the protein. Both heparin binding and hemagglutination are expressed by the N-terminal half of FHA, indicating that this domain contains important functional regions of the molecule.

Exported proteins in gram-negative microorganisms face the challenge of having to cross two distinct membranes, called the inner and outer membranes. In most cases, translocation through the inner membrane involves the signal peptide-dependent general secretion pathway (for a recent review, see reference 22). Important exceptions include the members of protein families related to the RTX hemolysins (31). Transport across the outer membrane often requires the concerted action of several accessory proteins that were thought to be specific for a given exported protein but that appear now to fall within distinct protein families.

Successful interaction of bacterial pathogens with their hosts usually requires the production of several extracellular proteins that constitute important virulence factors. Therefore, pathogenic organisms represent interesting models for the study of protein export, especially across the outer membrane. *Bordetella pertussis*, the etiologic agent of whooping cough, produces several virulence factors that are located at the outer surface of the organism or released into the extracellular milieu. These factors include pertussis toxin, adenylate cyclase toxin, filamentous hemagglutinin (FHA), fimbriae, and probably other proteins that have yet to be identified. Accessory genes important for the biogenesis and export of several of these factors have been isolated and studied (6, 14, 30, 32, 33). Interestingly, in each case the accessory proteins were found to be members of protein families involved in export and biogenesis of macromolecules in other bacterial genera.

Among the various protein export systems of *B. pertussis*, the FHA export machinery is particularly efficient, because FHA represents the major secreted protein in *B. pertussis* culture supernatants. This high efficiency is particularly interesting because of the large size of monomeric FHA polypeptides (220 kDa).

FHA is a major adhesin produced by several *Bordetella* species. It expresses at least three different adherence activities (for a recent review, see reference 13). The region downstream of the FHA structural gene (*fhaB*) contains several open reading frames, of which the three most proximal are involved in the biogenesis of the fimbriae (14, 32, 33) and the most distal is involved in the biogenesis of FHA (32). The protein encoded by this latter gene, named *fhaC*, has significant sequence similarities with accessory proteins involved in the secretion of hemolysins of *Serratia marcescens* (20) and *Proteus mirabilis* (27). In addition, the N-terminal region of FHA has strong sequence similarities with the N-terminal domains of the *S. marcescens* and *P. mirabilis* hemolysins (4). This region was found to be important for hemolysin (25) and FHA export (32), respectively, strongly suggesting that the hemolysin export systems have characteristics in common with the export system for FHA.

The open reading frame of the *fhaB* gene has the capacity to code for a 367-kDa protein, referred to as FhaB (4, 5), and the mature protein, referred to as FHA, represents the approximately 60% N-terminal portion of FhaB. The C-terminal part of FhaB is not present in the mature FHA but contains several particular features, such as a proline/lysine-rich domain, several proline-rich repeats, and an RGD sequence.

To assess the functional role of the C-terminal and N-terminal regions of FhaB, we set out to analyze their involvement in the biogenesis of FHA, using progressive deletions from the 3' end of *fhaB* toward its 5' end. The results indicate that this

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>B. pertussis</i>		
BPSM	Streptomycin- and nalidixic acid-resistant Tohama I derivative	17
BPGR4	10-kb <i>EcoRI</i> deletion of <i>fhaB</i>	14
BPGR7	0.45-kb <i>PstI</i> deletion in <i>fhaB</i>	32
BPMC	11.65-kb <i>EcoRI-SphI</i> deletion in <i>fhaB</i> and <i>fimA</i>	14
BPNJ4	Chromosomal insertion of pUC18EH in BPGR4	This study
<i>B. parapertussis</i>	Clinical isolate	18
<i>E. coli</i> JM109		34
Plasmids		
pBBR122	Chloramphenicol- and kanamycin-resistant pBBR1 derivative	1a
pBG1	10-kb <i>EcoRI</i> <i>fhaB</i> fragment in pBBR122	This study
pBG13	<i>BglII-BamHI</i> c deletion in pBG1	This study
pBG8	<i>BamHIIb-BamHIIc</i> deletion in pBG1	This study
pBG4	<i>BamHIIa-BamHIIc</i> deletion in pBG1	This study
pBG7	0.45-kb <i>PstI</i> deletion in <i>fhaB</i> in pBG4	This study
pBG12	<i>SalIa-BamHIIc</i> deletion in pBG4	This study
pBG15	1.9-kb <i>PvuI</i> deletion in pBG4	This study
pRIT13202	10-kb <i>EcoRI</i> <i>fhaB</i> fragment in pUC18	4
pRIT13197	<i>EcoRI-BglII</i> <i>fhaB</i> fragment in pUC8	4
pUC18	ColE1, ampicillin resistance	34
pUC18-3	1.25-kb <i>SphI-SalI</i> <i>fhaB</i> fragment in pUC18	This study
pUC18-4	0.45-kb <i>PstI</i> deletion in pUC18-3	This study
pUC18-5	<i>SalIa-BamHIIa</i> <i>fhaB</i> fragment in pUC18-4	This study
pGR5	5' end of <i>bvgAS</i> and 3' end of <i>fhaB</i> in pSS1129	14
pUC18EH	2.2-kb <i>EcoRI-HindIII</i> fragment of pGR5 in pUC18	This study

region is involved in the biogenesis of FHA, because deletions or frameshift mutations result in either no or highly reduced levels of FHA. However, further deletions toward the N terminus show that smaller peptides can be secreted at very high levels in an *fhaC*-dependent manner.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *B. pertussis* and *Bordetella parapertussis* strains were grown on Bordet-Gengou agar (3) supplemented with 1% glycerol and 20% defibrinated sheep blood or in modified Stainer and Scholte medium containing 1 g of 2,6-*O*-dimethyl- β -cyclodextrin (8) per liter and the relevant antibiotics at the following concentrations: streptomycin, 100 μ g/ml; gentamicin, 10 μ g/ml; and kanamycin, 10 μ g/ml. *Escherichia coli* strains were grown in LB broth (24), and antibiotic-resistant *E. coli* was selected with 100 μ g of ampicillin, 25 μ g of gentamicin, or 25 μ g of kanamycin per ml.

Recombinant DNA techniques. Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were purchased from Boehringer Mannheim and used as recommended by the supplier. Plasmid DNA was isolated by using either the rapid alkaline lysis method as described by Sambrook et al. (24) or disposable Qiagen columns (Cogen) under the conditions recommended by the supplier. DNA fragments were eluted from agarose gel with a GeneClean kit (Bio 101). All other DNA manipulations were performed as described by Sambrook et al. (24).

Constructions of plasmids containing various truncated *fhaB*-derived genes. To complement the *fhaB* deletion in *B. pertussis* BPGR4, we chose the kanamycin- and chloramphenicol-resistant plasmid pBBR122, a derivative of the broad-host-range plasmid pBBR1 isolated from *Bordetella bronchiseptica* by Antoine and Loch (1a). To create pBG1, the 10-kb *EcoRI* fragment containing the nearly entire *fhaB* gene, including its promoter and regulatory sites, was isolated from pRIT13202 (4) and inserted into the unique *EcoRI* site of pBBR122, located in the kanamycin resistance gene. pBG4 resulted from the digestion of pBG1 with *BamHI* and religation, thereby eliminating the two *BamHI* fragments of 2.37 and 4.7 kb. To generate pBG8, the 4.7-kb *BamHI* fragment isolated from pRIT13202 was inserted into the *BamHI* site of pBG4. pBG13 resulted from the exchange of the 2.5-kb *SphI-BamHI* fragment of pBG4 for the 6-kb *SphI-BglII* fragment of pRIT13202. To construct pBG7, the 1.27-kb *SphI-SalI* fragment isolated from pRIT13197 (4) was first subcloned into pUC18, previously digested with *SphI* and *SalI*, and the resulting plasmid, pUC18-3, was then digested with *PstI* and ligated to generate pUC18-4, missing the 471-bp *PstI* fragment. The 1.26-kb *BamHI-SalI* fragment isolated from pRIT13197 was then inserted into pUC18-4, previously digested with *BamHI* and *SalI*, to create pUC18-5, and the 2.1-kb *SphI-BamHI* fragment from pUC18-5 was finally introduced into pBG4, previ-

ously digested with *SphI* and *BamHI*. Consequently, pBG7 is similar to pBG4 except for the in-frame deletion of the 471-bp *PstI* fragment coding for the FHA region homologous to the N-terminal regions of ShlA and HpmA of *S. marcescens* and *P. mirabilis*, respectively. To create pBG12, the 2.5-kb *SphI-BamHI* fragment from pBG4 was exchanged for the 1.27-kb *SphI-BamHI* fragment isolated from pUC18-3. pBG15 was obtained after digestion of pBG4 with *PvuI* and ligation of the 3.65-kb *PvuI* fragment with the 2.76-kb *PvuI* fragment. To construct pUC18EH, the 2.17-kb *EcoRI-HindIII* fragment of pGR5 (14) was inserted into pUC18 digested with *EcoRI* and *HindIII*.

Electroporation of *B. pertussis* and *B. parapertussis*. The various pBBR122 derivatives described above were introduced into different *Bordetella* strains by electroporation as described elsewhere (1). Kanamycin-resistant *Bordetella* colonies were analyzed for the presence of the recombinant plasmids by the rapid electrotransformation method described by Baulard et al. (2) or directly by immunostaining for the presence of FHA derivatives.

Identification of FHA-related polypeptides. To detect FHA-related polypeptides, culture supernatant or cell-associated protein fractions of the different *Bordetella* cultures were analyzed by electrophoresis on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel as described by Laemmli (11) and Coomassie brilliant blue staining or by immunoblotting as described by Towbin et al. (26), using rat polyclonal antibodies directed against native FHA or truncated FHA (named Fha44) or using monoclonal antibody X3C (12), kindly provided by M. Brennan, or 12.2H5 (4), kindly provided by I. Heron. Immunoblot analyses were performed by using a Minifold II apparatus (Schleicher & Schuell) containing a nitrocellulose membrane and by serially diluting Fha44 or FHA twofold in phosphate-buffered saline (PBS), starting with a concentration of 125 or 500 μ g/ml, respectively. Each slot received 200 μ l. The antigen solutions were then aspirated, and the filters were washed several times with PBS containing 0.01% Tween 20 (PBS-Tween). The nitrocellulose membrane was then incubated with the anti-Fha44 antibodies for 2 h at room temperature, washed three times with PBS-Tween, incubated for 1 h with peroxidase-linked rabbit anti-rat antibodies (Diagnostic Pasteur, Marnes-la-Coquette, France), and finally developed with 4-chloro-1-naphthol (Bio-Rad) and hydrogen peroxide. Immunoblots and Coomassie blue-stained polyacrylamide gels were scanned with a Bio-Rad model GS-670 imaging densitometer, and the areas of the peaks were compared with each other on the same blot or gel to estimate the relative quantities or immunoreactivities of FHA and Fha44.

Polyclonal antisera. Rat polyclonal antibodies directed against purified FHA or Fha44 were obtained as described by Vaitukaitis et al. (28), using 50 μ g of FHA or Fha44. Three weeks after the primary immunization, two boosts with the same preparation were given intraperitoneally. The anti-FHA and anti-Fha44 sera were then enriched for specific antibodies after absorption of the nonspecific antibodies on a *B. pertussis* BPGR4 crude cell lysate.

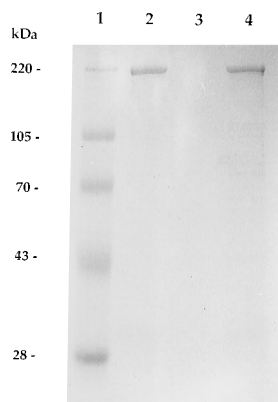


FIG. 1. SDS-PAGE analysis of *B. pertussis* BPSM, BPGR4, and BPGR4(pBG1) culture supernatants. One hundred-microliter aliquots of unconcentrated *B. pertussis* culture supernatants at stationary phase were analyzed by SDS-PAGE and Coomassie blue staining. The supernatants were from strains BPSM (lane 2), BPGR4 (lane 3), and BPGR4 containing pBG1 (lane 4). Lane 1, size markers (sizes are given at the left).

Hemagglutination assay. Fifty-microliter aliquots of twofold serial dilutions of FHA or Fha44, ranging from 50 to 0.5 $\mu\text{g/ml}$ in PBS, were each added to 50 μl of a 5% suspension of fresh rabbit erythrocytes in 96-well plastic plates. After 2 h of incubation at room temperature, the plates were read for hemagglutination activity.

Purification of Fha44 by heparin-Sepharose. FHA and the truncated FHA analog Fha44 were purified on heparin-Sepharose from strains BPSM and BPGR4(pBG4), respectively, as described for mature FHA by Menozzi et al. (16).

RESULTS

FHA complementation of *B. pertussis* BPGR4 with a pBBR1 derivative carrying the nearly complete *fhaB* gene. *B. pertussis* BPGR4 is a strain from which a 10-kb *EcoRI* fragment, containing the nearly entire *fhaB* gene, including its promoter and regulatory sites, was deleted (14). To analyze the role of the C-terminal domain of FhaB in FHA biogenesis, we developed a complementation system for this strain, using a derivative of pBBR1, a broad-host-range plasmid previously isolated from *B. bronchiseptica*. This derivative, named pBBR122, contains chloramphenicol and kanamycin resistance markers as described previously (1a). The 10-kb *EcoRI* fragment containing the complete *fhaB* sequence except for the last 990 bp of the open reading frame was cloned into the unique *EcoRI* site of pBBR122. The resulting plasmid, pBG1, was then introduced into *B. pertussis* BPGR4. Analysis of culture supernatants by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining revealed that BPGR4(pBG1) secreted FHA at a level similar to that of the wild-type strain BPSM, a streptomycin- and nalidixic acid-resistant Tohama I derivative (Fig. 1), whereas no FHA was found in untransformed BPGR4. Western blot (immunoblot) analysis using several anti-FHA monoclonal antibodies confirmed that the protein secreted by BPGR4(pBG1) was FHA (data not shown). This finding demonstrates that pBG1 can efficiently complement the *fhaB* mutation of BPGR4 in *trans* and that the last 330 amino acids of FhaB are not essential for the efficient production and secretion of FHA.

Role of the C-terminal domain of FhaB in the biogenesis of FHA and truncated analogs. To analyze the role of the C-terminal region of FhaB in the biogenesis of FHA and truncated analogs, different deletions of the 3' end of *fhaB* were constructed in the 10-kb *fhaB EcoRI* fragment of pBG1 as described in Materials and Methods. The resulting recombi-

nant plasmids, pBG13, pBG8, pBG4, pBG12, and pBG15, containing the various deletions as depicted in Fig. 2 were then introduced into BPGR4, and both the cells and the culture supernatants from each recombinant strain were analyzed by SDS-PAGE and Coomassie blue staining as well as by Western blotting with rat polyclonal antibodies directed against FHA.

As shown in Fig. 3, BPGR4(pBG13), containing a *BglII*-to-*BamHI*C deletion in *fhaB*, produced a truncated secreted protein with a size slightly smaller than that of FHA and reactive with anti-FHA polyclonal antibodies. Compared with BPGR4(pBG1) and the wild-type strain BPSM, BPGR4(pBG13) yielded significantly less truncated FHA in the culture supernatant (Fig. 3, lanes 2), indicating that efficient extracellular production of mature FHA requires the C-terminal domain of FhaB. On the other hand, the truncated FHA derivative is well produced in a cell-associated form (Fig. 2).

Similar to BPGR4(pBG13), BPGR4(pBG8), containing a *BamHIB*-to-*BamHIC* deletion in *fhaB*, also produced less FHA derivative than BPSM or BPGR4(pBG1). In contrast, BPGR4(pBG4), containing the *BamHIA*-to-*BamHIC* deletion in *fhaB*, produced very efficiently a secreted truncated FHA derivative (Fig. 3A). This truncated FHA polypeptide was named Fha44. The amounts of Fha44 found in the culture supernatants of BPGR4(pBG4) were significantly greater than those of FHA found in BPSM or BPGR4(pBG1) culture supernatants. Densitometric scanning of the Coomassie blue-stained gel indicated that on a molar basis, approximately sevenfold more Fha44 than FHA was secreted. This finding indicates that the first 947 amino acids of FHA do not require the C-terminal portion of FhaB for extracellular production. The culture supernatant of BPGR4(pBG4) also contained a second, smaller polypeptide that is most likely a degradation product of Fha44. Degradation products were also found in the case of the other constructs but were present at lower levels than the approximately 50% seen with Fha44.

To confirm that the greater extracellular production of Fha44 than of natural FHA was not due to a copy number effect of the pBBR122 derivatives, the truncated *fhaB* gene from pBG4 was subcloned into a pUC18 derivative, yielding pUC18EH, and introduced into the chromosome of BPGR4. This yielded *B. pertussis* BPNJ4, with a single truncated *fhaB* gene in the *fha* locus. SDS-PAGE analysis indicated that the production of the truncated FHA derivative in this strain was also significantly greater than the FHA production in BPSM.

As shown in Fig. 3B, Fha44 reacts very poorly with the polyclonal anti-FHA antiserum, whereas it reacts strongly with the anti-FHA monoclonal antibody X3C (data not shown), thereby confirming its identity as an FHA-related polypeptide. Densitometric scanning of the Western blot indicated that the polyclonal antibodies recognized Fha44 approximately 100-fold less well than FHA, as determined by a molar comparison (Fig. 4).

BPGR4(pBG12), containing the *SalI*-to-*BamHIC* deletion in *fhaB*, also produced relatively high levels of truncated extracellular FHA (Fig. 2). It was also readily detected by monoclonal antibody X3C. Finally, no FHA-related polypeptide could be detected in the culture supernatant of BPGR4(pBG15), containing the *PvuIIa*-to-*PvuIe* deletion in *fhaB*.

Together, these results indicate that the C-terminal domain of FhaB is required for efficient full-length FHA biogenesis, whereas it is not required for the extracellular production of truncated FHA derivatives, encompassing the N-terminal half of the mature protein. This finding implies that the N-terminal region of FHA contains all of the information necessary for its secretion.

Fha44 biogenesis requires expression of *fhaC*. The N-termi-

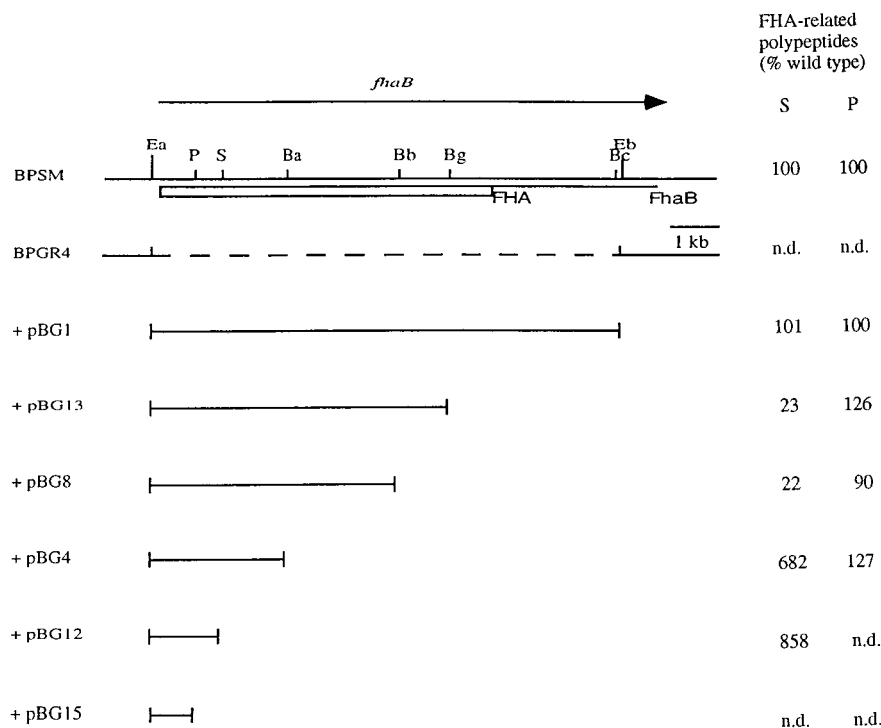


FIG. 2. Partial restriction map of *fhaB* and localization of the various deletions. The arrow indicates the length and direction of the *fhaB* gene. Strain BPSM contains the full-length *fhaB* gene in the chromosome. The open box (FHA) designates the coding region of the mature FHA protein, and the line (FhaB) designates the coding region of the FhaB precursor protein. The following restriction sites are indicated: Ba, Bb, and Bc, the first, second, and third *Bam*HI sites; Bg, *Bgl*II; Ea and Eb, the first and second *Eco*RI sites; P, the first *Pst*I site; S, the first *Sal*I site. Strain BPGR4 contains the Ea/Eb deletion, as indicated by the broken line. Plasmids pBG1, pBG13, pBG8, pBG4, pBG12, and pBG16 are pBBR122 derivatives containing the portions of the *fhaB* gene indicated by the horizontal lines. The production of secreted (S) or cell-associated (P) FHA analogs is given at the right as percent FHA polypeptides produced by the different strains compared with that produced by the wild-type strain BPSM (100% reference) on a molar basis. The material in the culture supernatant was quantified by densitometric scanning of Coomassie blue-stained gels. The cell-associated material was quantified by densitometric scanning of Western blots, using polyclonal anti-FHA or anti-Fha44 antibodies, in comparison with the scanning of Western blots containing secreted FHA polypeptides and probed with the same antibodies. n.d., not detectable.

nal domain of FHA contains sequence similarities with the N-terminal regions of the *S. marcescens* and *P. mirabilis* hemolysins ShlA and HpmA, respectively (4). Previous work has suggested that, like in the hemolysins, this region is involved in FHA biogenesis (32). The results described above indicate that the N-terminal domain of FHA that includes this homologous sequence is sufficient for efficient secretion of truncated FHA derivatives. Since FHA secretion depends on expression of *fhaC*, it was of interest to investigate whether the secretion of Fha44 also requires the expression of *fhaC*.

pBG4 was therefore introduced into *B. pertussis* BPMC, a strain lacking both the *fhaB* gene and the expression signals of *fhaC*. Consequently, this strain does not express *fhaB* or *fhaC* (14). As shown in Fig. 5, no Fha44 was detected in the BPMC(pBG4) culture supernatant after SDS-PAGE and Coomassie blue staining, indicating that secretion of Fha44 requires the expression of *fhaC*. This finding is consistent with the notion that the *fhaC* gene product interacts, directly or indirectly, with the N-terminal region of FHA and that this interaction is required for secretion of FHA into the culture supernatant, similar to what has been proposed to be the mechanism of ShlA secretion in *S. marcescens*.

To further investigate whether the presence of the N-terminal box homologous to the N-terminal part of ShlA is essential for the secretion of truncated FHA, we constructed pBG7, which corresponds to pBG4, containing an in-frame deletion of the 471-bp *Pst*I fragment encoding the homologous region. This plasmid was then introduced into BPGR4, and the result-

ing strain was found to not produce any detectable extracellular FHA-related polypeptides, as analyzed by SDS-PAGE and Coomassie blue staining of culture supernatants (Fig. 5). This result was very similar to those obtained earlier with the full-length mutant FHA in *B. pertussis* BPGR7 (32) and confirms the importance of the hemolysin-homologous sequences in FhaC-mediated FHA secretion.

Fha44 export in other *Bordetella* strains. To assess whether Fha44 can be produced and secreted by other *Bordetella* species, pBG4 was transferred into *B. parapertussis*. The culture supernatant of *B. parapertussis*(pBG4) was analyzed by SDS-PAGE and Western blotting (Fig. 6, lane 4); the results indicated that *B. parapertussis* is able to efficiently secrete Fha44, suggesting that *B. parapertussis* expresses an accessory gene similar to *fhaC*. However, similar to what is observed for full-length FHA, *B. parapertussis*(pBG4) produces somewhat less secreted Fha44 than *B. pertussis* BPSM(pBG4). Also, in contrast to *B. pertussis*(pBG4), Fha44 in the culture supernatants of *B. parapertussis*(pBG4) appeared to be much more stable, since the lower- M_r form was not detected in fresh culture supernatants.

When pBG4 was introduced into *B. pertussis* BPSM, both FHA and Fha44 were well secreted into the culture medium (Fig. 6, lane 5), demonstrating that the production and secretion of Fha44 do not affect the production and secretion of the natural FHA and reciprocally, and that therefore the export machinery in BPSM is not saturated by the natural *fhaB* gene expression.

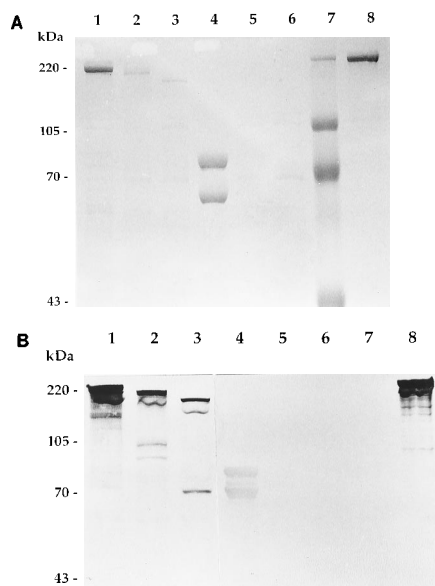


FIG. 3. SDS-PAGE and immunoblot analyses of *B. pertussis* strains containing various deletions in the 3' end of *fhaB*. One hundred-microliter aliquots of culture supernatants of the different *B. pertussis* strains were analyzed. Lanes 1 through 7 contain the supernatants of BPGR4 harboring pBG1 (lanes 1), pBG13 (lanes 2), pBG8 (lanes 3), pBG4 (lanes 4), pBG12 (lanes 5), and pBG15 (lanes 6). Lanes 8 contain culture supernatants from strain BPSM, and lanes 7 contain size markers, the sizes of which are given at the left. After electrophoresis, the gel was stained with Coomassie blue (A) or transferred to nitrocellulose and developed with anti-FHA polyclonal antibodies (B).

Functional roles of the N-terminal domain of FHA encoded by pBG4. Mature FHA from wild-type *B. pertussis* harbors at least three binding sites responsible for the bacterial attachment to different cell types (13). Two of these sites, an RGD sequence responsible for binding to CR3 integrins on macro-

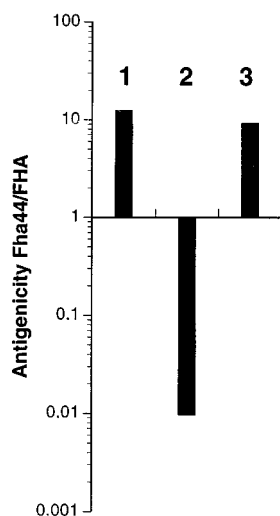


FIG. 4. Relative immunoreactivities of FHA and Fha44 with various antibodies. Densitometric scanning was applied on immunoblot blots containing FHA and Fha44 incubated with monoclonal antibody 12.2H5 (column 1), anti-FHA polyclonal antibodies (column 2), or anti-Fha44 polyclonal antibodies (column 3). After scanning, the areas (intensity and width of the peaks) corresponding to the immunoreactivity of Fha44 were compared with those corresponding to the immunoreactivity of FHA on a molar basis, and the results are expressed as immunoreactive ratios of Fha44 to FHA.

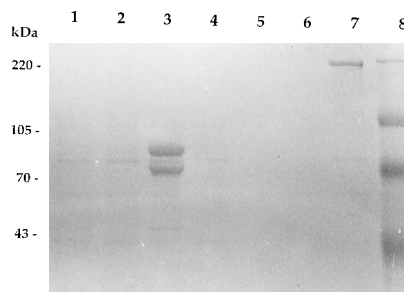


FIG. 5. SDS-PAGE analysis of several *B. pertussis* culture supernatants. One hundred-microliter aliquots of culture supernatants from BPGR7 (lane 1), BPMC(pBG4) (lane 2), BPGR4(pBG4) (lane 3), BPMC (lane 4), BPGR4(pBG7) (lane 5), BPGR4 (lane 6), and BPSM (lane 7) were analyzed by SDS-PAGE. The size markers are shown in lane 8. After electrophoresis, the gel was stained with Coomassie blue. The sizes of the markers are given at the left.

phages and a carbohydrate-binding site responsible for binding to epithelial cilia, have previously been mapped outside the Fha44 domain (21, 23). The heparin-binding site has not been precisely mapped but was suggested to be close to the N-terminal region of FHA (7, 17).

To characterize the heparin-binding property of Fha44 and to compare it with that of mature FHA produced by BPSM, the Fha44 molecule was purified on heparin-Sepharose. As described for the purification of FHA by Menozzi et al. (16), a culture supernatant from BPGR4(pBG4) was directly applied onto the heparin-Sepharose matrix. Fha44 was completely retained on the column and was eluted from the matrix by 300 mM NaCl, similar to what has been observed previously for full-length FHA. This result demonstrates that the N-terminal portion of FHA contains the heparin-binding site present in native FHA.

The hemagglutination property of purified Fha44 was also examined and compared with that of purified full-length FHA. The results demonstrate that the N-terminal portion of FHA retains the ability to hemagglutinate sheep erythrocytes. However, at the same concentration, the hemagglutination activity obtained with purified Fha44 was weaker than obtained with native FHA, suggesting that other regions in native FHA are also involved in this activity or that the folding of Fha44 is sufficiently different from that of native FHA to interfere somewhat with hemagglutination.

Antigenicity and immunogenicity of Fha44. Mature FHA

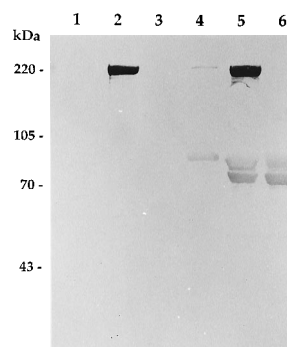


FIG. 6. Immunoblot analysis of *Bordetella* culture supernatants. Culture supernatants from *B. pertussis* BPSM (lane 2), BPGR4 (lane 3), BPSM(pBG4) (lane 5), and BPGR4(pBG4) (lane 6) and from *B. parapertussis*(pBG4) were analyzed by immunoblotting using polyclonal anti-FHA antibodies. The numbers at the left indicate the sizes of the markers.

contains an immunodominant region localized at the C terminus of the mature protein. As shown above, anti-FHA antibodies recognize Fha44 very weakly, in agreement with previous results (4). Quantification of the immunoblots indicated that the reactivity of anti-FHA antibodies was approximately 100-fold weaker against Fha44 than against FHA (Fig. 4). Hyperimmunized animals, however, are able to produce antibodies that also recognize the N-terminal region (12), indicating that it is not devoid of epitopes. Nevertheless, this finding suggests either that the N-terminal region of FHA is not very immunogenic or that the immunodominant part of the molecule somehow masks the N-terminal domain.

To distinguish between these two possibilities, rats were immunized with heparin-purified Fha44, and the rat anti-Fha44 antibodies were analyzed for their recognition of Fha44 and native FHA by quantitative slot blot analysis. As shown in Fig. 4, immunization with Fha44 induces antibodies that recognize mainly Fha44 and, more poorly (approximately 10-fold less), native FHA. This finding indicates that the isolated N-terminal portion of FHA is also immunogenic and suggests that this region is masked by the C-terminal immunodominant part in full-length FHA.

Differential reactivity of monoclonal antibody 12.2H5 further defines one of the N-terminal regions of FHA that are masked by the C-terminal domain. Epitope mapping using recombinant FHA fragments fused to the *E. coli* maltose-binding protein have indicated that the 12.2H5 epitope is located in the domain that contains sequence similarities with the *S. marcescens* and *P. mirabilis* hemolysins (11a). This antibody was found to react more strongly with the Fha44 polypeptide than with full-length FHA (Fig. 4), consistent with the notion that its epitope is located in the N-terminal domain but that it is somewhat masked by the C-terminal domain.

DISCUSSION

FHA is the major secreted protein produced by *B. pertussis*. The mature form has a molecular mass of 220 kDa and is a filamentous molecule approximately 2 nm wide and 50 nm long (for a recent review, see reference 13) folded into a monomeric hairpin (15). Despite its size, it is extremely efficiently secreted into the extracellular milieu. FHA secretion requires at least one accessory protein, encoded by *fhaC*, a gene located 3' of the structural *fhaB* gene within a polycistronic operon that also contains accessory genes necessary for the biogenesis of *B. pertussis* fimbriae (32). The N-terminal region of FHA contains approximately 100 amino acid residues homologous to the *S. marcescens* and *P. mirabilis* calcium-independent hemolysins (4). As with these hemolysins, this domain is thought to play an important role in the secretion of FHA, because an in-frame deletion of this region completely abolishes FHA biogenesis (32). The current model implies interaction of this N-terminal domain of FHA with FhaC, located in the outer membrane. We have recently been able to secrete FHA peptides from *E. coli* strains that also express *fhaC* in *trans* (9), suggesting that no additional *B. pertussis*-specific factor is required for FHA export through the outer membrane and that the FHA-FhaC interaction may be direct.

In this study, we found that the C-terminal domain of the FHA precursor FhaB is important for efficient secretion of full-length FHA but not for the secretion of truncated FHA derivatives of smaller size (80 kDa and less). According to the three-dimensional model recently proposed by Makhov et al. (15) on the basis of ultrastructural analysis of purified FHA, these smaller FHA analogs make up only part of one side of the FHA hairpin rod. The secretion of these smaller polypep-

tides still depends on FhaC and on the N-terminal region of FHA that is homologous to the *S. marcescens* and *P. mirabilis* hemolysins.

Several other virulence factors produced by gram-negative pathogens have also been shown to undergo significant C-terminal truncations of their precursors during biogenesis. However, in these cases, the C-terminal domain of the precursor plays an active role in targeting the protein for secretion, as exemplified by the immunoglobulin A protease of *Neisseria gonorrhoeae* (19). This domain inserts into the outer membrane and probably serves as an essential channel for the translocation of the protein through the membrane. Because of the very high secretion efficiency of Fha44 and because of the diminished but still detectable secretion of longer FHA analogs, the C-terminal domain of FhaB must play a different role in FHA biogenesis.

Polyclonal anti-FHA antibodies were found to poorly recognize Fha44, a C-terminally truncated FHA polypeptide, and conversely, polyclonal anti-Fha44 antibodies poorly recognize full-length mature FHA. These immunogenicity and antigenicity studies of full-length and truncated FHA molecules suggest that the N-terminal domain of mature FHA is largely masked by its C-terminal domain. Antigenicity studies with monoclonal antibody 12.2H5 further indicated that at least the part containing the domain with sequence similarities to the *S. marcescens* and *P. mirabilis* hemolysins is masked in the mature protein. This observation is consistent with the rigid hairpin rod model proposed by Makhov et al. (15). The masking of the N-terminal region by the C-terminal domain might render the former domain unavailable for productive interaction of mature FHA with another protein such as FhaC. Obviously, in the case of Fha44, the N-terminal region of FHA is not masked by the C-terminal domain and consequently would be free to interact with FhaC during the export process. Therefore, it is conceivable that the role of the C-terminal domain of FhaB is to prevent the premature formation of the rigid hairpin. This role as an intramolecular chaperone could be achieved by protein-protein interactions between the C-terminal domain of FhaB and that of FHA within the same molecule, as proposed in the working model depicted in Fig. 7. This would keep the N-terminal domain of FHA free to interact with FhaC during biogenesis. Once the FHA part of FhaB reaches the outside of the bacterial cell, the C-terminal domain of FhaB is removed and the hairpin of the mature molecule can be formed spontaneously. Such an intramolecular chaperone activity of the C-terminal domain of the precursor on the C-terminal domain of the mature part of the protein would explain the requirement of the C-terminal domain of FhaB for efficient secretion of full-length FHA and larger truncated forms but not for that of smaller truncated forms, as found in this study. Of course, it may also be possible that the polypeptides encoded by pBG13 and pBG8 are more susceptible to degradation than FHA or Fha44. Although this possibility cannot be ruled out at this point, it should be noted that the amounts of cell-associated FHA-related polypeptides were roughly similar in BPGR4 strains containing either pBG1, pBG13, or pBG8 (Fig. 2), and there was no evidence of more degradation in BPGR4(pBG13) or BPGR4(pBG8) than in BPGR4(pBG1).

Chaperones are commonly involved in protein transport across membranes. In the general secretion pathway through the bacterial cytoplasmic membrane, the SecB molecular chaperone is required to retard folding of secreted proteins (22). The formation of Sec-dependent type 1 or Pap pili requires additional, periplasmic chaperones that prevent premature periplasmic aggregation of pilin subunits prior to their incorporation into pilus structures (10). Sec-independent secretion

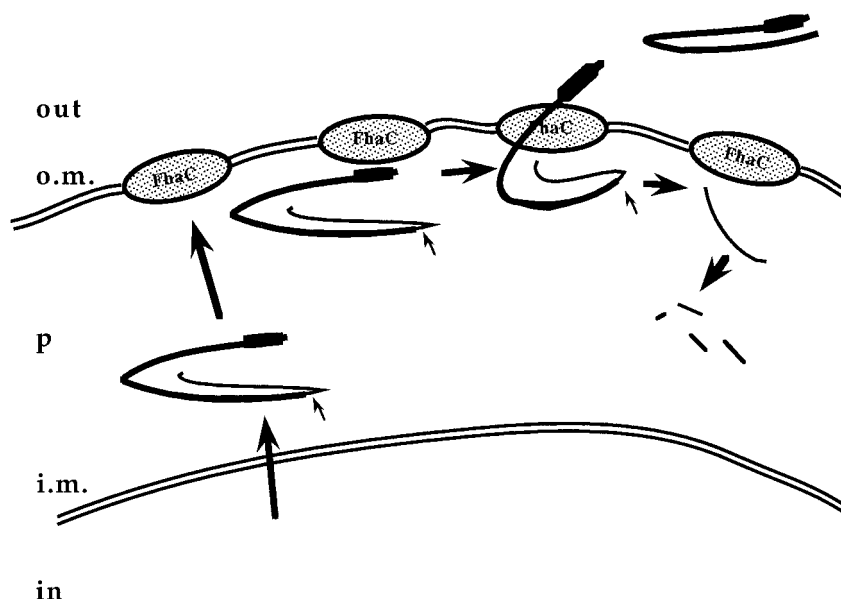


FIG. 7. Model for a potential role of the C-terminal domain of FhaB. The bacterial cell envelope is represented by the inner membrane (i.m.), periplasm (p), and the outer membrane (o.m.). FhaC is shown embedded in the outer membrane as a stippled oval. in and out designate the inside and outside of the cell, respectively. The proposed trafficking of FhaA during its biogenesis is indicated by the large arrows. FhaA and FhaB are represented by the black line containing a black box at one end. The FhaA portion of FhaB is indicated by the thickened line. This box represents the N-terminal sequence homologous to the *S. marcescens* and *P. mirabilis* hemolysins. The small arrows indicate the cleavage site of FhaB to generate FhaA. After its translocation through the inner membrane, the C-terminal domain of FhaB covers the C-terminal half of the mature portion of the protein, thereby leaving the N-terminal domain free to interact with FhaC. Upon interaction with FhaC, the N-terminal domain crosses the outer membrane first, followed by the C-terminal domain of the mature portion. Finally, the C-terminal domain of the precursor is cleaved off, allowing mature FhaA to fold into its hairpin structure at the outside of the cell. The C-terminal domain of FhaB stays in the cell and is eventually degraded.

of the *Yersinia* Yop proteins also requires chaperones, called Syc proteins, to prevent premature folding of the Yop proteins (29). In all of these cases, however, and in contrast to the proposed Fha model, the chaperones are distinct molecules and not translationally fused to their protein substrates. Interestingly, the Yop chaperones contain leucine-rich motifs resembling leucine zippers. Leucine-rich repeats are also found in mature Fha (15) and can be extended into the C-terminal domain of FhaB (12a). The leucine-rich repeats of mature Fha have been proposed to play a role in the formation of the hairpin structure (15). It is therefore not unreasonable to speculate that the chaperone mechanism needed to prevent premature hairpin formation similarly involves the leucine-rich repeats. We are currently testing the intramolecular chaperone model for the C-terminal domain of FhaB through protein-protein interaction studies and genetic complementation.

Whatever the role played by the C-terminal domain of FhaB, it apparently does not require the last 330 amino acid residues. This extreme C-terminal domain of FhaB contains proline-rich sequences and the (PK)₅ peptide. This sequence has previously been proposed to anchor Fha within the cell envelope, perhaps by spanning the periplasmic space and linking the inner and outer membranes (5). Obviously, the contributions of these structures to the biogenesis of Fha are at most only minor.

Not all of the activities of the N-terminal domain of Fha appeared to be masked by its C-terminal domain. Fha44 was found to bind to heparin-Sepharose in a similar fashion as full-length Fha. Both can be eluted by approximately 300 mM NaCl, suggesting that their affinities for heparin are very similar. This finding indicates that in full-length Fha, the N-terminal heparin-binding site is not masked by the C-terminal domain of the protein. It also confirms our previous studies (7), suggesting that the heparin-binding domain of Fha is

located in the N-terminal half of the molecule. The hemagglutination activity of Fha was previously suggested to involve its heparin-binding site (16). This notion was confirmed in this study by the finding that Fha44 is able to hemagglutinate rabbit erythrocytes. Thus, both the heparin-binding and the hemagglutination activities involving the N-terminal domain of Fha are not inhibited by the hairpin folding of the complete protein.

Because the genetic constructs described in this report do not insert an in-frame stop codon at the precise site of gene truncation, 43 amino acid residues were added to the Fha-related peptides. This addition does apparently not dramatically affect the secretion of the Fha polypeptide, which suggests that the Fha secretion mechanism can be used to carry foreign proteins or peptides along with Fha. The binding activity of Fha44 to heparin provides a convenient purification method for such recombinant fusion proteins. Work is in progress to evaluate the potential of the Fha secretion mechanism for the export of foreign proteins either in *B. pertussis* or in other gram-negative organisms.

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